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Honors Thesis Project in Biology

Pcdh19 Expression in Normal and Regenerating Adult Zebrafish Retinas

Kristin Forkapa, with Dr. Qin Liu

Introduction

My Honors Research Project is to examine protocadherin-19 mRNA (*pcdh19*) expression in normal and regenerating adult zebrafish (*Danio rerio*) retinas. Pcdh19 is a member of the cadherin superfamily (Vanhalst et al., 2005). Cadherins are cell adhesion molecules important for animal development and maintenance of adult structures (Takeichi, 1990). *pcdh19* is expressed mainly in the central nervous system (CNS) of developing vertebrates (Liu et al., 2010), but there is little information on its expression in adult organisms (Liu et al., 2015). In mammals, injuries to the central nervous system (CNS), including the optic nerve, usually result in permanent damages due to intrinsic factors and environmental factors. The intrinsic factors include increased expression of genes (e.g. growth promoting genes such as GAP43, transcription factors such as Klf7a, Pax6 and Vsx-1) that promote neuronal survival and axonal outgrowth. There is little or no such increased gene expression in the mammalian CNS after damages (Apara 2014). The environmental factors include expression of inhibitory molecules (e.g. nogo) and formation of scar tissues in injured sites (Apara et al., 2014). Unlike mammals, fish and amphibians have the ability to regenerate their optic nerves after damages (Sperry, 1948). Following an optic nerve lesion (cut or crush) in adult zebrafish, most retinal ganglion cells (their axons join together to form the optic nerve) survive, and their regenerating axons grow out of the retina and reach to anterior part of the optic tectum (the major brain target of the retinal ganglion cells) in one week (Zou, 2013). The regenerating retinal axons cover the entire tectal surface in about three weeks, and refine their retino-tectal synapses in the next 4-8 weeks (Zou et al., 2013). Molecular mechanisms underlying the optic nerve regeneration are still under intense investigation. Studies in Dr. Liu's laboratory showed that expression of several cadherins, including N-cadherin (also called cadherin-2), was greatly increased during adult

zebrafish optic nerve regeneration (Liu et al., 2002), suggesting that cadherins may be involved in the optic nerve regeneration. This idea is supported by preliminary results recently obtained in Dr. Liu's laboratory showing that blocking N-cadherin function using electroporation of a zebrafish N-cadherin specific morpholino antisense oligonucleotide (NcadMO) severely affected the optic nerve regeneration (personal communication with Dr. Liu). Cadherins affect animal development by affecting cell-cell adhesion and expression and function of other molecules (reviewed by Cavallaro and Dejana, 2011). As the first step in determining pcdh19 function in the optic nerve regeneration, I examined its mRNA expression in adult zebrafish retinas with optic nerve crushed, and compared it to control retinas with no optic nerve damages. I also examined expression of zebrafish Klf7a mRNA (*klf7a*) in the retinas, because Klf7a, a transcription factor, is a known marker for the regenerating adult zebrafish retina, and shown to play an important role in the optic nerve regeneration (Veldman et al., 2007).

Materials and Methods

Reagents

1. Alkaline phosphatase substrate: dissolve 1.75g Tris-HCl in 950 ml distilled water, pH to 7.5.
2. Blocking solution: 20 ml 10% Blocking reagent (Roche, Indianapolis, IN), 1 ml 10% Triton X-100 (Fisher Scientific, Waltham, MA), 179 ml 2X SSC (Sigma, St. Louis, MO).
3. Genius Buffer 3: 20 ml 10X Tris/NaCl stock solution + 160 ml distilled water + 20 ml 10X MgCl₂ (Fisher Scientific) stock solution.
4. Hybridization solution: 36 µl TEN buffer, 250 µl formamide (Roche), 100 µl 50% dextran sulfate (Sigma, 50% in DEPC water), 50 µl 10% blocking solution, 1.5-2 µg DIG RNA probe, X µl DEPC water to make a final volume of 500 µl.

5. Maleate buffer: made from 5X Maleate buffer (58 g Maleic acid (Fisher Scientific) in 850 ml distilled water, pH to 7.5 using a lot of NaOH pellets, add 43.8g NaCl, bring up to 1L with distilled water).
6. Proteinase K solution: Add 250 μ l Proteinase K (Roche, 10 mg/ml stock) to 250 ml proteinase K buffer [25ml 1M Tris (Sigma, pH 8.0) + 25ml 0.5M EDTA (Sigma, pH 8.0) + 150 ml DEPC water].
7. TEN buffer: 5 ml 1M Tris-HCl (Sigma), pH 7.5, 30 ml 5M NaCl (Fisher Scientific), 1 ml 0.5M EDTA.
8. TEA (triethanolamine) solution: 9.3g TEA (Sigma) in 490 ml DEPC water and pH to 8.0 using 11-12 NaOH tablets (Fisher Scientific), add DEPC water to make 500 ml.
9. RNase buffer: 29.23g NaCl, 10 ml 1M Tris (pH 7.5), 2 ml 0.5M EDTA, bring up to 1L with distilled water.
10. 10X $MgCl_2$ stock: Dissolve 50.8g $MgCl$ (Fisher Scientific) in 420 ml of distilled water. Bring up to 500 ml and sterile filter into a very clean bottle.
11. 10X Tris/NaCl stock: 78.5g TrisHCl (Sigma) dissolved in 350ml distilled water + 29g NaCl. pH to 9.5 using a lot of NaOH pellets. Bring volume to 500 ml. Sterile filter into a very clean bottle.

Animals and surgery

Retinal tissues: fixed zebrafish retinal tissues were provided by Dr. Qin Liu. Adult zebrafish (*Danio rerio*) were raised from embryos obtained from breeding wildtype zebrafish housed in the University of Akron vivarium. All animal related procedures were approved by the Institutional Animal Care and Use Committee (IACUC). The fish were kept in 10-gallon glass

tanks at a temperature around 28.5°C, with 12-hour light/12-hour dark. Optic nerve crushes were performed by Dr. Liu according to procedures described in Liu et al. (2002). Briefly, fish were anesthetized in 0.03% tricaine ethanesulfonate (also called MS-222, Sigma). Each fish was placed in a 60 mm plastic petri-dish, wrapped in a piece of wet paper towel with the head exposed and the left side up. The posterior side of the left eye was partially pulled out to expose the optic nerve. Under a dissecting scope, the exposed optic nerve was crushed using a pair of fine-tipped forceps. The fish was returned to a fish tank and allowed to survive for various periods (e.g. 1-7 days) before being sacrificed for collecting retinal tissues. There were six animals for each survival group (a total of 18 fish).

Tissue fixation, cryoprotection and sectioning

To harvest the retinal tissues, the fish were anesthetized with MS-222, placed in the petri-dish half filled with ice. After removing the lens from each eye, the ocular muscles and the optic nerve were cut with a pair of small surgical scissors. The eyes, both experimental (with optic nerve lesioned) and control (without the optic nerve crush) eyes were quickly placed (separately) in ice-cold 4% paraformaldehyde solution (paraformaldehyde, Fisher Scientific, dissolved in phosphate buffered saline, PBS, pH=7.4). The eyes were fixed overnight at 4°C. The next morning, the eyes were washed three times, 10 min. each in PBS at room temperature, followed by immersing in 20% sucrose (also in PBS) overnight at 4°C. The next morning, the eyes were placed in a mixture (1:1) of 20% sucrose and Optimal Compound Tissue (OCT, Fisher Scientific) for one hour with constant agitation (on a slow moving shaker) before they were embedded in the same mixture (3 eyes in each tissue block), frozen using dry ice + 95% ethanol.

The frozen tissue blocks were placed in a labeled-biopsy bag, stapled shut, and placed in a -80°C freezer until use.

The eyes were sectioned at 14 µm using a cryostat. The tissue sections were placed on Fisher Superfrost plus glass slides (Fisher Scientific). The slides were dried at room temperature for one hour before stored in a -20°C freezer until used for in situ hybridization (see below).

In situ hybridization

Detailed procedures for in situ hybridization (ISH) on tissue sections were described in Liu et al. (1999). The slides were removed from the freezer and allowed to dry in room temperature for 20-30 minutes. While the slides were drying, the TEA solution (see Reagents) was made and a proteinase K (see Reagents) solution was prepared and warmed to 37°C. The slides were rehydrated in a series of decreasing concentrations of ethanol solutions (100% (twice), 95%, 70%), and then in 2X SSC (saline-sodium citrate)- all one min. each. Next, the slides were incubated in the pre-warmed proteinase K solution for 3 min., followed by a rinse in DEPC water at room temperature. Next the tissue sections were rinsed in the TEA solution for 3 min. Afterward, the sections were incubated for 10 min. in a mixture of 650 µl acetic anhydride and 250 ml TEA. The slides were dehydrated in a series of increasing concentrations of ethanol (70%, 95%, and 100% (twice)). The slides were then drained and left to dry at room temperature for one hour.

While the tissue slides were drying, the ISH probe was prepared. The zebrafish digoxigenin-labeled antisense *pcdh19* cRNA probe was produced by Dr. Liu and stored at -80°C. Once it was removed from the -80°C freezer, the probe was thawed on ice for about 5 min., and then placed in a 68-70°C water bath for 10 min. Quickly following that, the probe was placed

on ice for 2 min. (the heating and cooling are necessary to keep the probe from forming secondary structures that would interfere with binding to its target mRNA). The probe was quickly spun in a centrifuge before putting it back on ice. Next, the probe was added to the hybridization solution at a concentration of 2.5 µg/ml and mixed well. Last, the tissue sections were covered with 65-75 µl of hybridization solution on RNase free siliconized HybridSlips (Sigma) and placed in a hybridization oven at 58°C overnight.

After the hybridization, the slides were treated for post-hybridization washes. The slides were washed in 2X SSC for 30 min. at 37°C, followed by an one hour wash in 50% formamide (Sigma) in 2X SSC at 58°C. The tissue slides were then incubated in a 400 µl RNase A (Sigma, 10 mg/ml) solution for 30 min. at 37°C. This was followed by two 2X SSC washes at 37°C. The tissue slides were then placed in an RNase buffer (without RNase A) at 65°C for 30 min.

The final part of the ISH experiment was the ICC detection. To do this, the tissue sections were washed in a blocking solution (2X SSC + 1% blocking reagent + 0.05% Triton X-100) for 2-3 hours at room temperature on a shaker. After this, the sections were washed in 1X Maleate buffer, twice, for 5 min. each at room temperature. A 2-2.5 µl anti-DIG-Alkaline Phosphatase antibody (Roche) was added to 5 ml of blocking solution (1X Maleate buffer, 500 µl of 10% blocking reagent (Roche) and supplemented with 150 µl 10% Triton X-100, and mixed well. 200 µl of the solution was added to each CoverWell (Fisher Scientific, with the CoverWell upside up to cover the sections). Then, the sections were incubated overnight in a humid chamber at 4°C. The next morning, the CoverWell was removed and the sections were washed twice (10 min. each) in 1X Maleate buffer. The following wash was in Genius Buffer 3 for 10 min. Then, one NBT/BCIP ready-to-use tablet (Roche) was added to 10 ml distilled water and allowed to dissolve completely (by vortex). The sections were incubated in 200 µl of the solution

(CoverWell upside down) for overnight at room temperature. Afterwards, the sections were washed in an alkaline phosphatase substrate for 30 min. at room temperature. Lastly, the sections were coverslipped with 100% glycerol (Fisher Scientific) and sealed at the edges with Permount (Fisher Scientific).

Data collection

The processed tissue sections were viewed under a compound microscope (Olympus BX51) equipped with a SPOT digital camera system (Diagnostic Instrument Inc., Sterling Heights, MI). Digital images were taken of the tissue samples and processed with Adobe Photoshop (San Jose, CA).

Results

Expression of *pcdh19* and *klf7a* in both control and lesioned retinas was examined one day, three days and one week after the optic nerve crush. All twelve retinas (six from control side, and six from lesioned side) from one day after the optic nerve crush were used, and they had similar expression patterns for both *klf7a* and *pcdh19* within each group. In the control (uninjured) retinas, there was little *klf7a* expression in the retinal ganglion cell layer (GCL, Fig. 1A), while apparent *pcdh19* was expressed in both the GCL and the inner half of the inner nuclear layer (INL, Fig. 1B). Similar *klf7a* expression was observed in the lesioned retina (Fig. 1C), except that there appeared to be a slight increase in *klf7a* expression in the GCL. Expression of *pcdh19* was decreased in the GCL of the lesioned retina (Fig. 1D), while *pcdh19* expression in the INL appeared to be similar to that in the control retina.

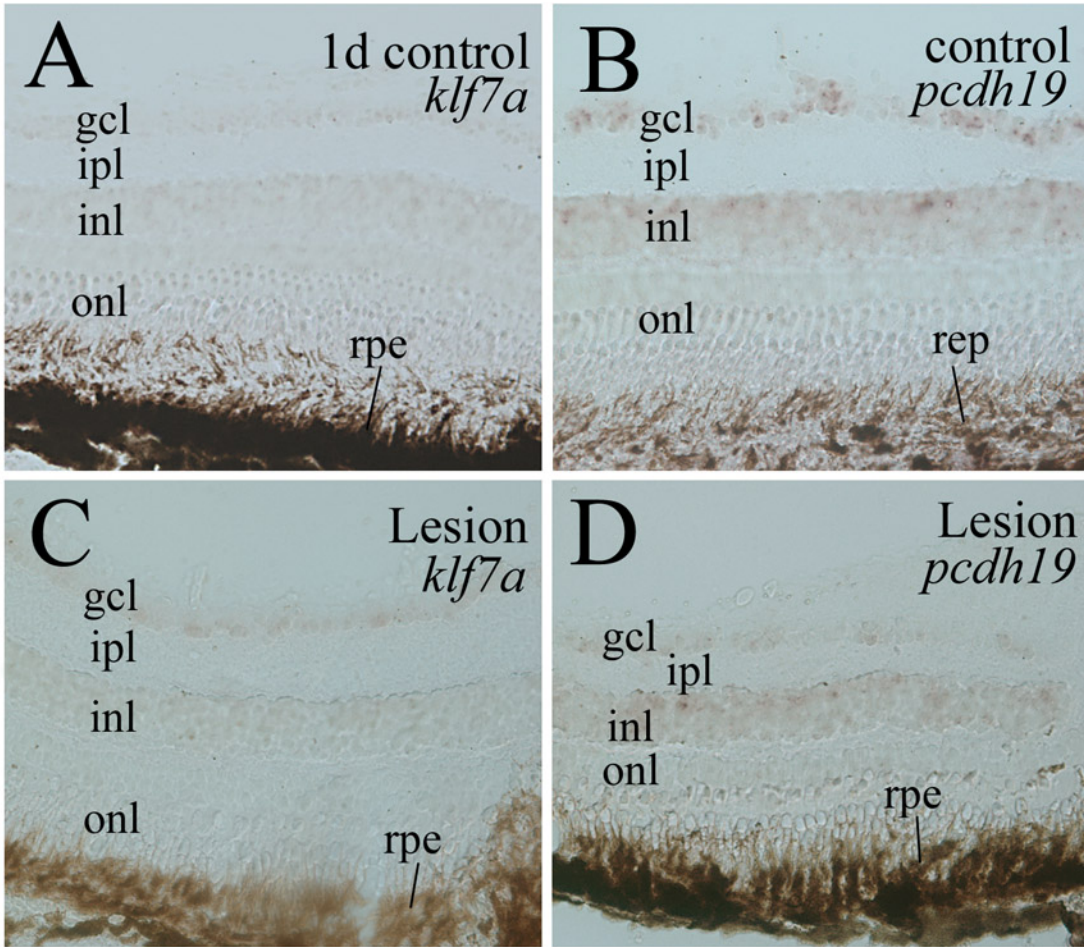


Figure 1. *klf7a* and *pcdh19* expression in a control retina (panels A and B) and a retina one day after the optic nerve crush (panels C and D). Abbreviations: gcl, retinal ganglion cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; onl, outer nuclear layer; rpe, retinal pigmented epithelium.

In fish three days following the optic nerve crush, data was collected from six retinas from the control side, and five retinas from the lesioned side, because one eye from the lesioned side appeared to be degenerating (smaller and darker with opaque lens), therefore was not used. All control retinas had similar expression patterns for both *klf7a* and *pcdh19*, while all lesioned retinas showed similar *klf7a* and *pcdh19* expression. There was little *klf7a* expression in the GCL

of the control retina (Fig. 2A). However, in the lesioned retina (Fig. 1C), *klf7a* was strongly expressed in the GCL, as previously demonstrated by Veldman and colleagues (2007). (add a space)*pcdh19* expression in the control retina (Fig. 2B) was similar to its expression in one day control retina (Fig. 1B), with obvious expression in both the GCL and the inner half of the INL. Expression of *pcdh19* was decreased in the GCL of the lesioned retina (Fig. 2D), which was also similar to *pcdh19* expression in the lesioned retina of one day following the optic nerve crush (see above).

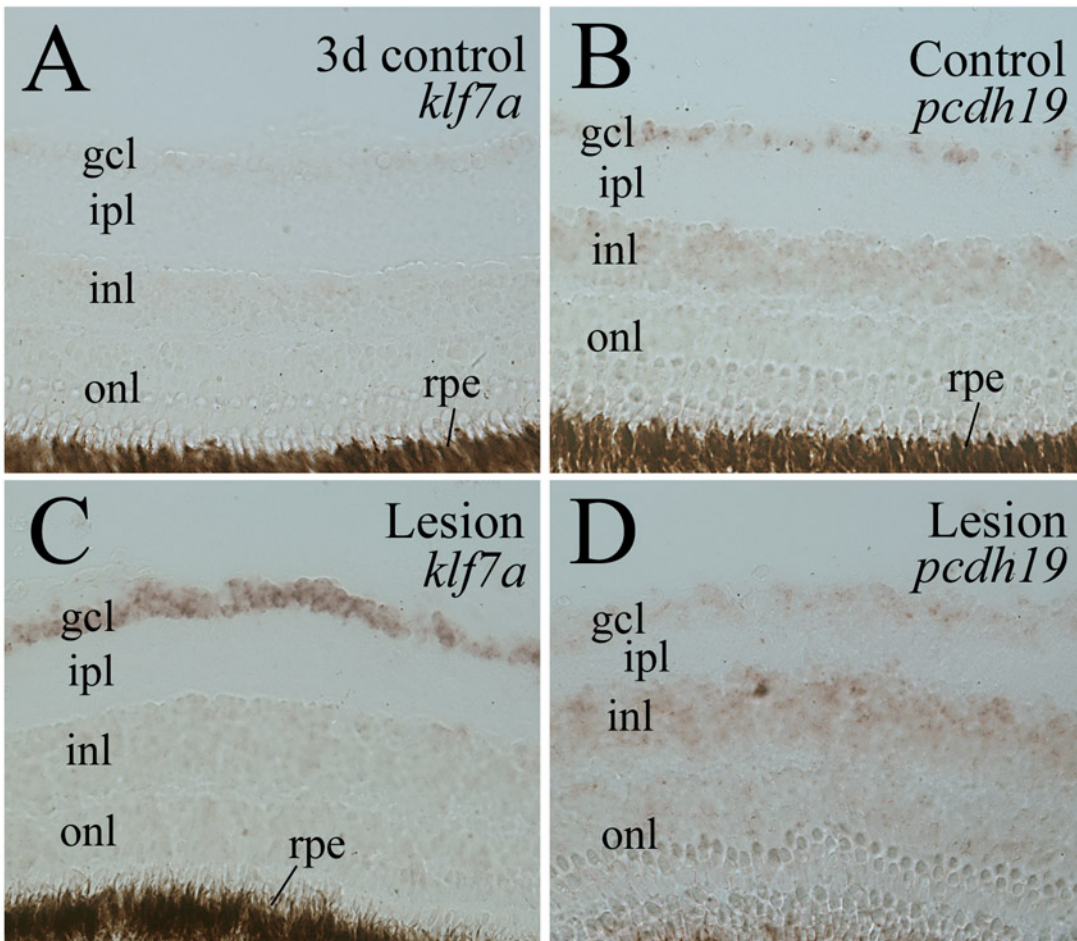


Figure 2. *klf7a* and *pcdh19* expression in a control retina (panels A and B) and a retina three days after the optic nerve crush (panels C and D). Abbreviations are the same as in Figure 1.

One week following the optic nerve crush, the *klf7a* expression in the GCL of the control retina remained the same as the control retinas of one- and three-day post optic nerve crush animals (Fig. 2A). Also similar to the lesioned retina three days after the lesion (Fig. 2C), *klf7a* expression in the GCL was greatly increased one week after the optic nerve crush (Fig. 3C). *pcdh19* expression in the control retina one week after the lesion (Fig. 3B) was similar to its expression in the control retinas of one- and three-day lesioned fish (see above): it was expressed in both in the GCL and the inner half of the INL. Expression of *pcdh19* in the lesioned retina (Fig. 3D) continued to be decreased compared to the control retina, but appeared to be slightly increased compared to the lesioned retinas of one- and three-day following the optic nerve crush (see above).

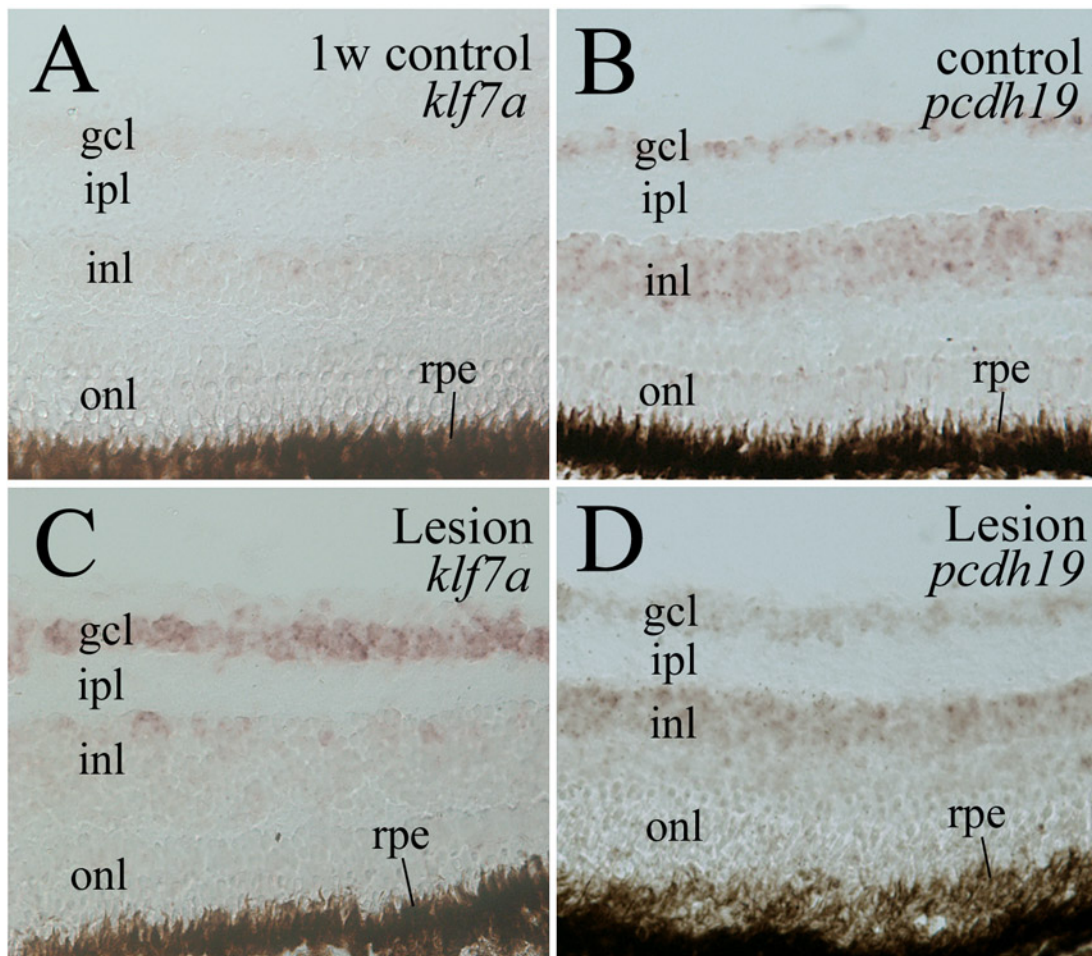


Figure 3. *klf7a* and *pcdh19* expression in a control retina (panels A and B) and a retina one week (1 w) after the optic nerve crush (panels C and D). Abbreviations are the same as in Figure 1.

The above results from fish 1 week after the optic nerve crush were obtained from six retinas from the control side, and five retinas from the lesioned side (one eye on the lesioned side was missing from a fish). Again, all six control retinas had similar *klf7a* and *pcdh19* expression patterns, while the two genes showed similar expression patterns in all five regenerating retinas.

Discussion

My results of *klf7a* expression in the control and lesioned retinas of one-day, three-day and one week following the optic nerve lesion were consistent with findings from Veldman et al. (2007), using ISH for three-day lesioned tissues, and real-time PCR for one-day and one week tissues, showing that there was little *klf7a* expression in the control retinas, while its expression was greatly increased in the lesioned retinas. Therefore I am confident that my results of *pcdh19* expression in the control and regenerating adult zebrafish retinas are reliable. Persistent strong *pcdh19* expression in normal adult zebrafish retina is a little surprising, because most cadherins show strong expression only in embryonic retinal tissues, and their expression become much reduced in adult retinas (Liu et al., 2001). In fact, *pcdh19* expression domain in the adult zebrafish retina is wider (in both GCL and INL) than its expression domain (only in the GCL) in retinas 2- and 3-day old zebrafish embryos (Liu et al., 2010). In the adult fish retina, I showed that changed *pcdh19* expression was mainly confined to the GCL of the regenerating retina, which is not surprising because crushing the optic nerve produces damages to the axons of retinal ganglion cells that reside almost exclusively in the GCL. Another unexpected result is that *pcdh19* expression is apparently decreased in the GCL of the regenerating retina, because

previous studies by Liu and colleagues showed that N-cadherin and R-cadherin expression were both greatly increased in regenerating adult zebrafish retinas (Liu et al., 2002). The authors also showed that expression of these two cadherins was also increased in the regenerating adult zebrafish cerebellum (Liu et al., 2004). It is therefore possible that some cadherins (e.g. cadherin-2 and cadherin-4) promote retinal axon regrowth, while others (e.g. pcdh19) inhibit retinal axon outgrowth. Preliminary results in Dr. Liu's laboratory on cadherin-2 function in the optic nerve regeneration support this idea. Future functional studies are needed to determine functions of pcdh19 and other cadherins in the optic nerve regeneration.

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